MONITORING OF GENOTOXICITY OF FORMALDEHYDE ON HUMAN BUCCAL CELLS WITH COMET ASSAY

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ABSTRACT
Purpose: Whether formaldehyde induces DNA breakage or not is still in debate. So far it is not clear whether dose-effect between formaldehyde and DNA breakage takes place while the DPC exists. Approach: In this study we had chosen human buccal cells as materials to explore the genotoxicity of liquid and gaseous formaldehyde with comet assay. Findings: The results showed that liquid formaldehyde induced DNA breakage in the range from 5 μM to 10 μM ($P<0.05$, compared with control group), and 7.5 μM was found to induce DNA breakage most. The DNA crosslinks were significantly found at 15 μM, 30 μM and 50 μM ($P<0.01$, compared with 7.5 μM group). DNA breakage caused by gaseous formaldehyde was found at 0.5 mg/m^3 and 1.0 mg/m^3 ($P<0.01$, compared with control group) but DNA crosslinks at 3.0 mg/m^3 significantly. Conclusions: The results suggest that formaldehyde induces DNA breakage in a very low concentration range but induces DNA crosslinks in a higher range.

INDEX TERMS
Formaldehyde, Genotoxicity, DNA breakage, DPC, Comet assay

INTRODUCTION
Formaldehyde (CAS No.50-0-0) is a colorless, highly flammable gas. Nowadays, people pay more attention on the indoor air pollutions. And formaldehyde is regarded as an important indoor air chemical pollutant for its extensive sources, high level indoors, long exposure duration and high toxicity. The elevated concentrations of formaldehyde in indoor air mainly emitted from the building materials, especially from wood-based panels. Many studies have shown that formaldehyde induced several diseases on human, even at fairly low level (0.5 mg/m^3) formaldehyde may induce eyes and upper respiratory irritancy (JRC 1990), occupational and environmental exposure at high level of gaseous formaldehyde increases the risks of asthma (WHO 2002). The results of large numbers of in vitro experiments with a variety of end-points indicate that formaldehyde is genotoxic at high concentrations in both bacterial and mammalian cells. It ranks in AI (carcinogenic to humans) of chemical (WHO 2004). The spectrum of mutation induced by formaldehyde in vitro varies among cell types and concentrations to which cells were exposed but includes both point and large-scale base mutation. Formaldehyde induced in vitro DNA-protein crosslinks (DPC, DPX), chromosomal aberrations, and sister chromatid exchange and gene mutations in rodent and human cells (WHO 2002), in which DPC initiate DNA replication error, resulting mutation. Therefore DPC has been recognized as a molecular bio-marker of potential mutation. Studies have indicated that formaldehyde induced DNA-protein crosslinks (Merk O & Speit G 1998, Speit G et al 2000, Speit G & Merk O 2002, Conaway CC et al, 1996), but it is not sure whether formaldehyde causes DNA strands breakage. The dose-effect between formaldehyde and DNA breakage while the DPC exists is not clear too.

The comet assay, also called single cell gel electrophoresis (SCGE), is a sensitive method to investigate DNA breakage in individual cells as a consequence of their in vitro or in vivo exposure to genotoxin. The method was first described by Östling and Johanson (1984) in 1984, and now it is also widely used to detect DNA-DNA or DNA-protein crosslinks by retardation in the rate of DNA migration (Tice RR 2000).

This study focuses on the genotoxicity of FA on human buccal cells with comet assay and the comparison of effects between the gaseous and liquid FA. The results suggest that FA induces both of the DNA strands breaks and the DNA-DNA or DNA-protein crosslinks. We also proposed a concept of "peak point" concentration of DNA breakage and found that the "peak point" of FA is about 7.5 μM. In this paper we have tried to use the "peak point" concept for statistically testing the DPC detection.

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RESEARCH METHODS

Cells And Chemicals

Buccal cells were obtained from a healthy human individual by scrapping the internal parts of the cheek with a toothbrush. The cells were added into 10 mL 0.9% NaCl and centrifuged at 6000 g for 2 min (Kaya et al 2002, Rojas E et al 1996). Formaldehyde was purchased from Sigma dissolved in dH2O immediately before use. Gaseous FA was generated by a small-scale environmental chamber (WH-II, Wuhan Yuxin Lit.) with wood-based panels in it. Low melting agarose (LMA) and normal melting agarose (NMA) were purchased from Promega. Acredine Orange (A.O) was purchased from Amresco.

Cell Treatment

Buccal cells were treated with 0 μM, 5 μM, 7.5 μM, 10 μM, 15 μM, 30 μM and 50 μM of liquid FA at 37℃ for 0.5 h or exposed to 0 mg/m^3, 0.5 mg/m^3, 1.0 mg/m^3 and 3.0 mg/m^3 of gaseous FA at 37℃ for 1 h, and then they were resuspended in 80 μL LMA.

Comet Assay

Comet assay was performed as described by Tice R.R. (2002) with some modification. After lysis (2.5 mM NaCl, 100 mM EDTA, 10 mM Tris-HCl [pH=10], 1% Triton X-100, and 10% DMSO) at 4 ℃ for 2 h, the slides were treated with 140 μl of proteinase K (10 mg/ml) at 37 ℃ for 2 h. Then the slides were placed in alkaline solution (300 mM NaOH, 1 mM EDTA, pH=13) for 20 min, and electrophoresis was conducted for 20 min at 17 V, and 240mA. All technical steps were conducted using very dim indirect light. After electrophoresis, the slides were neutralized with 0.4 M tris-HCl, pH 7.5 for three times for 5min, and then stained with A. O (20 μg/L) for 3 min. 50 cells in two slides were analyzed by the use of fluorescent microscope (WH-2, Olympus) and image analysis software (CASP, from www.casp.of.pl). Tail moment and tail DNA% was taken as the parameter of DNA damage (Tice RR 2000).

Water-Solubility Test

0.9 % NaCl were exposed to 0 mg/m^3, 0.5 mg/m^3, 1.0 mg/m^3 and 3.0 mg/m^3 of gaseous FA at 37℃ for 1 h. Meanwhile, the gaseous FA concentrations from the inlet and outlet of a small exposure chamber at 0 min, 30 min and 60 min were measured by the Interscan 4160 digital compact portable analyzer (USA). After exposure, the concentration of FA in NaCl solutions was tested with AHMT method immediately (Quesenberry MS & Lee YC 1996).

Statistical Analysis

All tests had been repeated in independent experiments. Differences between the groups and other values were tested for significance using Student's test (SigmPlot 2000 SPSS Inc.).

RESULTS

Liquid formaldehyde led to a strongly increased tail moment and tail DNA% at 5 μM, 7.5 μM and 10 μM ($P<0.01$) (Fig. 1 shows a comet image). However, FA caused a concentration-related decrease of tail moment and tail DNA% in DNA migration at 15 μM, 30 μM and 50 μM. The results suggest that FA induce DNA breakage in a very low concentration range but induce DNA crosslinks in higher concentrations, and 7.5 μM of FA induced DNA breakage most (Fig. 2). Therefore we regard 7.5 μM of FA as the peak point concentration of DNA breakage.

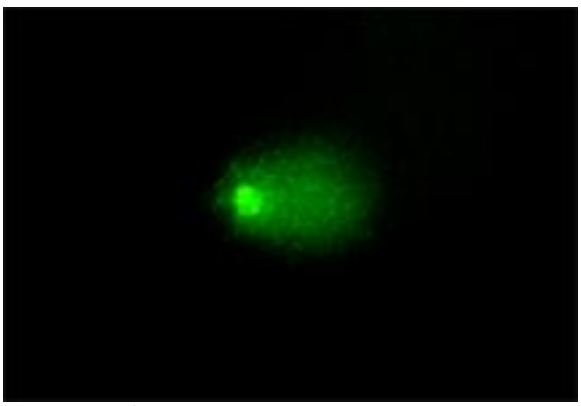

Figure 1. *The comet image of a human buccal cell treated with low concentrations of FA*

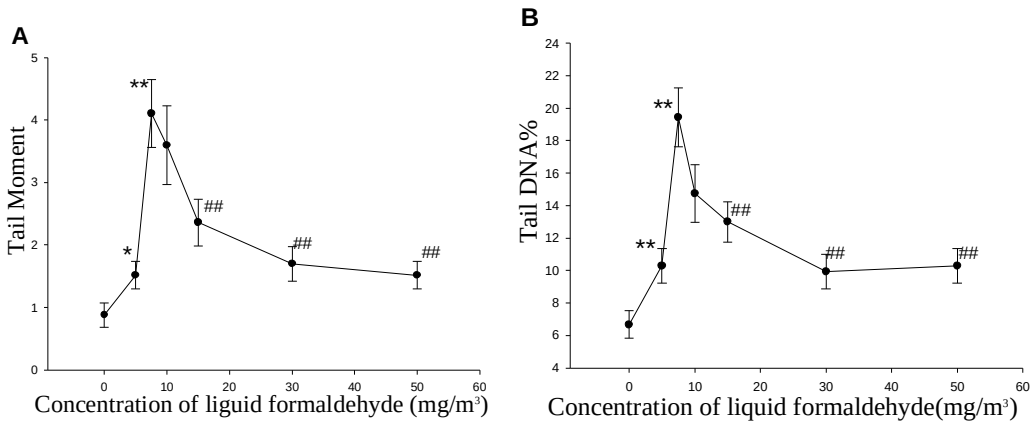

Figure 2. *The test results of comet assay of human buccal cells exposed to liquid formaldehyde (mean±SEM; *: P<0.05, **: P<0.01, compared with control group; ##: P<0.01, compared with 7.5 μM group)*

Table 1 shows the measurement results of FA concentrations from chamber emission. These measurements (0.47±0.05 mg/m^3, 1.00±0.04 mg/m^3 and 2.99±0.19 mg/m^3) were very close to the planed concentrations (0.5 mg/m^3, 1.0 mg/m^3 and 3.0 mg/m^3). It indicated that the gaseous FA from chamber emission is quite steady and reliable for exposure test.

In order to evaluate the solubility of gaseous FA, we measured the concentrations of FA in 0.9 % NaCl solutions. The results show in Table 2. The solubility of gaseous FA increased in a concentration-related way. At four different concentrations of gaseous FA (0.5 mg/m^3, 1.0 mg/m^3, 3.0 mg/m^3), the end concentrations in NaCl solutions are 4.85 μM, 9.40 μM, and 23.89 μM. We also calculated the solubility coefficient (SC) as follow:

$$SC = \frac{\text{FA concentrations in NaCl solution}(mg/m^3)}{\text{Gaseous FA concentrations } (mg/m^3)} \quad (1)$$

Table 1. *The measurement results of formaldehyde concentrations from chamber emission*

Time (min)	Concentration of FA（mg/m^3）		
	First treatment	Second treatment	Third treatment
0	0.525	1.038	2.975
30	0.450	1.013	2.800
60	0.438	0.963	3.188
Mean±S.D	0.47±0.05	1.00±0.04	2.99±0.19

Table 2. *The measurement results of formaldehyde concentrations in 0.9% NaCl solution*

Run	Concentration of FA（mg/L）		
	0.5 mg/m^3 group	1.0 mg/m^3 group	3.0 mg/m^3 group
A	0.155	0.287	0.727
B	0.139	0.282	0.721
Mean	0.147	0.285	0.724

Figure 3 shows the effects of gaseous formaldehyde-treatment on buccal cells. At 0.5 mg/m^3, tail moment and tail DNA% increased significantly compared with control (P<0.01). 1.0 mg/m^3 of FA induce the reduction of DNA migration but tail moment and tail DNA% is still significantly higher than control (P<0.01). However, when the concentration up to 3.0 mg/m^3 the tail moment and tail DNA% have no significant with control.

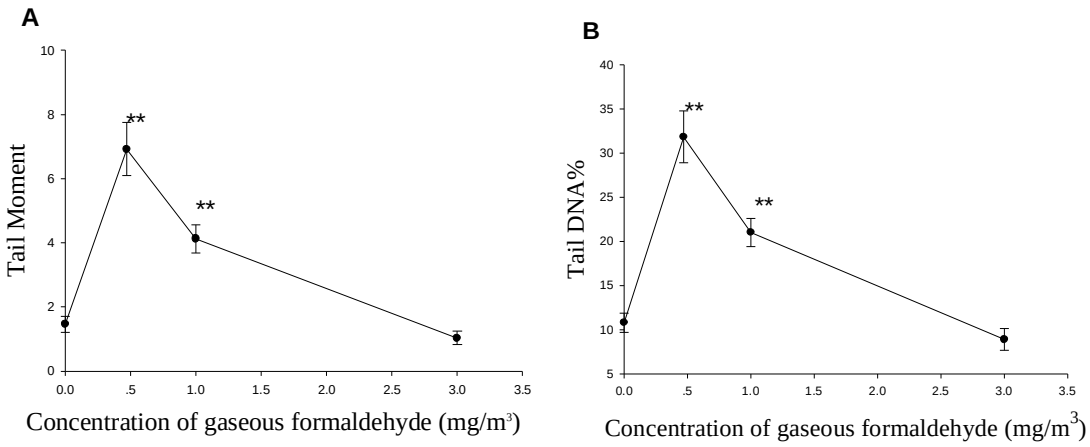

Figure 3. *The test results of comet assay of human buccal cells exposed to gaseous formaldehyde (mean±SEM; **: P<0.01, compared with control group)*

DISCUSSION

Studies indicated that tumors induced by FA are usually observed in the organs and tissues exposed to gaseous FA, and only in a higher concentration range (≥6 ppm) in which the increased DNA crosslinks are found clearly (WHO 2002, Conaway CC et al 1996). Owing to its reactivity with biological macromolecules, most of the FA is deposited and absorbed in the epithelia of upper respiratory track with which the substance comes into first contact. On the other hand, the cells at the exposed sites are more sensitive than other cells, and buccal mucosa cells are right at the exposed sites. Therefore buccal cell is one of the target cells to FA. It is the key of success that we preferred buccal cells as the experimental materials.

FA induces DNA-DNA and DNA-protein crosslinks in many types of cells (Merk O & Speit G 1998, Speit G et al 2000, Speit G & Merk O 2002). Studies indicated that FA mediates the form of covalent bond between lysine and base. The DNA-DNA crosslinks caused by FA are own to the reactivity of G, C and A bases. The discovery of 8-OHdG and methylene bis-adenosine in the exposed individual partly explains the mechanisms on genotoxicity of FA. Whether formaldehyde induces DNA breakage or not is still in debate. And it is not clear the dose-effect relationship between formaldehyde and DNA breakage while the DPC exists. Oliver Merk and associations have taken many experiments on the genotoxicity of FA, but they did not find the DNA breakage caused by FA. In China, scholars have studied the FA, and most of them even thought that FA does not induce DNA breakage. However, Tang Guohui and Xi Zhuge's studies showed that FA caused DNA strands breakage at 5 μM on HL60 cell line and at 10 μM on hepatic cells of rats (Tang GH et al 1997, Yang DF et al 2000). In this study, we found that FA induced DNA breakage in the range from 5μM to 10μM too, but when the concentration is up to 15μM the DNA crosslink effect caused by FA overlays DNA breakage. Our results indicate that FA induced DNA breakage occurs at low concentrations but DNA crosslinks at higher concentrations in a concentration-depend way. Reviewed the studies on FA, we find that most of the scholars had chosen the concentrations above 50 μM. So they thought FA had no effect on inducing DNA breakage.

Although FA can lead to DNA strands breaks and DNA crosslinks, it is not clear what the peak point concentration at which DNA breakage switch to DNA crosslinks is. In this study we found that FA caused DNA breakage at 5μM, 7.5 μM and 10 μM. FA induced DNA strands breaks most at 7.5 μM. So we suggest the peak point concentration is about 7.5 μM, and so far as we know, it is the first report on the peak point breakage concentration of FA. The discovery of it may make people evaluate genotoxicity of FA more correctly. We also propose that "peak point" may be used as a control base for statistically testing the crosslinks.

In order to compare the genotoxicity of gaseous and liquid FA, we explored the effect of gaseous FA on buccal cells. In vivo, gaseous FA does not directly act on cells. It solutes in body fluid， then liquid FA affect on different kinds of cells. We studied the solubility of gaseous FA, so that we could evaluate the changes of concentrations in this process. Results showed that the concentrations of FA in NaCl solution increased in a dose-depend way. And the solubility coefficients of them were changed between 241 and 294. After treated by gaseous FA at 0.5mg/m^3 and 1.0 mg/m^3 the end concentrations of solutions were 4.85μM and 9.40μM, and the DNA migration of buccal cells increased. But after treated by gaseous FA at 3.0mg/m^3 the end concentration of the solution was 23.89 μM, and the DNA migration of buccal cells decreased. It is consistent with our study on liquid FA. 4.85μM and 9.40μM

are in the DNA breakage concentration range, and 23.89 μM is in the DNA crosslinks concentration range. Our study gave a method to compare the concentrations of gaseous and liquid FA, and established a relationship on genotoxicity between gaseous and liquid FA.

The mechanisms of genotoxicity of FA are not clear. In our previous studies, we found that FA reduced the activity of superoxide dismutase (SOD) in different tissues. Others' studies showed that GSH enter into the metabolism of FA, and FA reduced the level of glutathione (GSH) in vivo (Teng S et al 2001). The decreasing of activity of SOD and level of GSH make reactive oxygen clear slowly, so the cells will be damage.

CONCLUSION

In conclusion, formaldehyde induced both DPC and DNA breakage, and 7.5 μM of liquid FA was found to induce DNA breakage most. Our results also indicate that comet assay, using buccal cells, could be a good biomarker of early effects of FA, and can be utilized for human monitoring and may be utilized for epidemiological studies.

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